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Composition of Honey. IV. Identification of the Disaccharides

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INTRODUCTION

Until recently the carbohydrates of honey were considered to be a simple mixture of glucose, fructose, and sucrose, with small amounts of a poorly characterized higher weight substance called honey dextrin. The reported occurrence of maltose (1) was confirmed by a fermentation procedure (2). Since the advent of chromatography, four articles have described chromatographic studies on honey carbohydrates. These have been reviewed in detail elsewhere (3). In all cases identification was based only upon R_f values, reaction to spray reagents, and, in one case (4), enzyme reactions. These latter investigators reported the presence in honey, in addition to glucose and fructose, of sucrose, maltose, isomaltose, erlose, kestose, melezitose, raffinose, dextrantriose, 4-glucosyldextrantriose, and a higher oligosaccharide. In none of this work were compounds isolated and no physical properties were determined.

We wish to report the isolation and identification of six disaccharides from honey. They were isolated by preparative paper chromatography and gradient elution from stearic acid-treated charcoal columns. They were identified by comparison of the infrared spectra of the free sugars and of their β -octaacetates with those of known carbohydrates. Several unresolved fractions were shown by zone electrophoresis of their borate complexes to contain an additional twelve compounds.

EXPERIMENTAL

Materials and Methods

Honey. All work was carried out with one lot of honey from Spanish needle (*Bidens aristosa*) from Arkansas. Charcoal column chromatography on Darco G-60-Celite²

¹ For Part III, see *Arch. Biochem. Biophys.* **79**, 165 (1959).

² Mention of trade names does not imply endorsement by the Department over others of a similar nature not named.

was done as described by Whistler and Durso (5). Whatman papers Nos. 1 and 3MM were used for chromatography. Irrigation solvent was 1-propanol-ethyl acetate-water, 7:1:2 (6). Presence of reducing sugar on papergrams was shown by reaction to triphenyltetrazolium chloride reagent (7) and of ketose by reaction to naphthoresorcinol (8). The aniline-diphenylamine reagent described by Harris and MacWilliam (9) was also used.

Zone electrophoresis was carried out on Whatman 3MM paper in 0.02 *M* sodium tetraborate (10) on apparatus similar to that of Kunkel and Tiselius (11).

RESULTS AND DISCUSSION

Preliminary Separation

A column (36 × 160 mm.) of charcoal-Celite was used to separate the disaccharide fraction (1.77 g.) from 16.1 g. honey. Paper chromatography showed it apparently to contain five components. Preparative paper chromatography on Whatman 3MM paper sheets (300 mg./sheet) yielded the fractions shown in Table I.

Further work on these fractions included determination of reducing value before and after various hydrolytic treatments. It became apparent that the fractions were considerably contaminated with nonsugar material, probably originating in the filter paper. Subsequently all paper used for isolative chromatography was washed with water on a Büchner-type filter made to hold full sheets (46 × 57 cm.).

Gradient Elution of Honey Disaccharides

A preliminary investigation of the value of gradient elution of disaccharides from stearic acid-treated charcoal with aqueous ethanol (12) showed that several sugar pairs inseparable by paper chromatography could be resolved. Accordingly 50 mg. of the mixed disaccharide fraction from honey was subjected to gradient elution from a 22 × 235 mm. stearic acid-treated charcoal column. Figure 1 shows the composition of the eluate. Sugar in each fraction was determined by an anthrone procedure (12). The results of chromatographic examination of various fractions is also shown. Table II gives the properties of these fractions.

TABLE I
Preliminary Fractionation of Honey Disaccharides

No.	Type	R_s^a	Yield
			mg.
1	Reducing aldose	0.56	66
2	Reducing aldose	0.70	92
3	Reducing ketose	0.83	85
4	Reducing ketose	0.99	47
5	Reducing ketose	1.19	13

^a R_s is migration referred to sucrose.

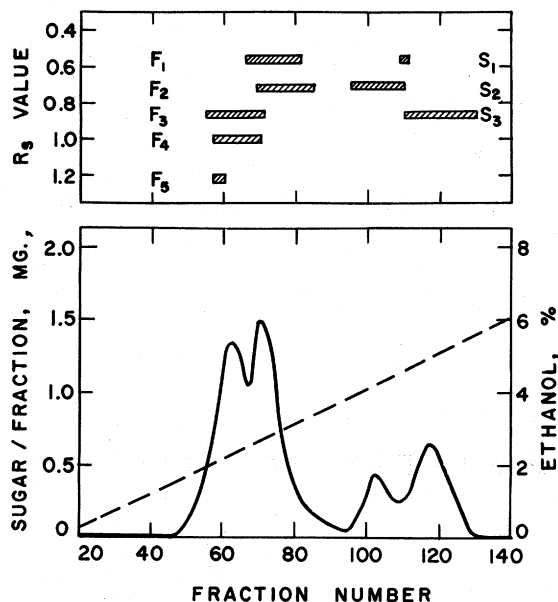


FIG. 1. Gradient-elution chromatography of honey disaccharides by aqueous ethanol from stearic acid-treated charcoal. Top: emergence of disaccharide fractions and their relative R_f values on paper. Bottom: sugar content (legend at left) and alcohol content (broken line) (legend at right) of effluent.

TABLE II
Disaccharides from Honey by Gradient Elution

No.	R_f	Type
F_1	0.57	Reducing aldose
F_2	0.73	Reducing ketose
F_3	0.86	Reducing ketose
F_4	1.00	Reducing ketose
F_5	1.22	Reducing ketose
S_1	0.62	Reducing aldose
S_2	0.70	Reducing aldose
S_3	0.87	Reducing aldose

Sugars labeled F were in the first group to emerge (Fig. 1), while those labeled S were in the slower group. Examination of the R_f values shows that three pairs of sugars (F_1 - S_1 , F_2 - S_2 , and F_3 - S_3) are inseparable by paper chromatography. Although tubes 95-105 appear from the figure to contain only S_2 , they actually contained small amounts of adjacent sugars. No tube contained only one sugar; hence paper chromatography and gradient elution were necessary for the separation.

TABLE III
Separated Honey Disaccharide Fractions

No.	Yield	Color reaction ^a
	<i>mg.</i>	
F_1	107	Gray
F_2	30	Yellow-brown
F_3	51	Purple
F_4	52	Rose
F_5	20	Yellow-brown
S_1	17	Gray
S_2	75	Blue
S_3	77	Gray

^a With aniline-diphenylamine reagent.

Repeated gradient-column separations were made, using a total of 2.6 g. of mixed disaccharides. Tubes corresponding to 50–80 (Fig. 1) were combined to make the F fraction (579 mg.); 100–130 for the S fraction (346 mg.). Each of these fractions was then resolved by preparative paper chromatography at 150 mg./sheet using water-washed paper. Repeated development³ was used to obtain optimum band separation. Use of the aniline-diphenylamine reagent facilitated observation of the separating sugars, since no adjacent sugars gave the same color reaction. Repeated papergram treatment was needed to separate fractions F_2 and F_3 . Table III shows the over-all yields and the color with the above reagent.

Infrared Identification of Isolated Disaccharides

A 10-mg. sample of each fraction from Table III was converted to the β -octaacetate as previously described (14). The infrared spectra of the carbohydrate and of the acetate were determined and compared with the spectra of known disaccharides and their acetates (14). In this way fractions F_1 , F_3 , and F_4 were identified, respectively, as isomaltose, maltulose, and turanose since the correspondence of the spectra of the free sugars and their acetates was exact in all respects. The spectra of several of the other nonacetylated fractions (F_5 , S_2 , and S_3) showed a prominent band at about 1600 cm^{-1} which was not present in the spectra of known sugars. As a band assigned to ionized carboxyl is in this region, the presence of stearic acid from the charcoal column was suspected. This possibility was eliminated by comparison of the spectrum of the residue from a blank

³ Papers were irrigated 16 hr., dried, and reirrigated. This was repeated as necessary. It differs from multiple development in that solvent is permitted to drip from the end of the paper; hence the compressive effect on zone separation after they pass the center of the sheet is avoided, while excessive broadening of bands during development is suppressed.

run through the column with that of stearic acid. Then the washed filter paper as used in the isolations was extracted with water in the amounts used. After evaporation the spectrum of the residue showed prominent bands at 1610 and 1405 cm^{-1} ; otherwise it was generally similar to carbohydrate spectra. This suggests that the material is that extracted from filter paper by Huffman *et al.* (15). They noted that repeated water washing failed to remove it completely from paper, and classified it as a hemicellulose on the basis of its composition.

This impurity is largely removed from disaccharide material by charcoal column adsorption. Hence by reversing the order of the isolation steps, i.e., paper chromatography followed by gradient elution of each fraction, these disaccharide fractions were obtained with greatly reduced contamination from the filter paper.

Although all spectra showed a minor absorption at 1650–1600 cm^{-1} , there was no interference with identification, which was based on the 650–1500 cm^{-1} region. Fractions S_2 and S_3 were identified, after reisolation, as maltose and nigerose, respectively. All bands of both the free sugars and of their β -octaacetates corresponded exactly with those of the known sugars and acetates. Fractions F_2 , F_5 , and S_1 have not yet been identified.

Paper Electrophoresis of the Disaccharide Fractions

The fractions listed in Table IV were subjected to paper electrophoresis in 0.02 *M* sodium tetraborate. Dried papers were treated with the color reagents described above, as well as with 0.016% bromothymol blue in 25% alcohol, which reveals nonreducing sugars in the presence of borate. Fractions F_1 , F_3 , F_4 , and S_3 showed single spots moving with the corresponding sugar borate. Fraction S_2 showed two components, the major migrating with the maltose complex, and one in small amount responding only to the indicator which moved with the trehalose complex on electrophoresis. Fraction F_5 contained one major and two minor components; F_2 showed five components: a nonreducing aldose, a reducing aldose, and three nonreducing ketoses. The first migrated with α,α -trehalose. Fraction S_1 contained four components in about equal amounts.

TABLE IV
Disaccharides Identified in Honey

Designation		
F_1	Isomaltose	6- <i>O</i> - α -D-Glucopyranosyl-D-glucose
F_3	Maltulose	4- <i>O</i> - α -D-Glucopyranosyl-D-fructose
F_4	Turanose	3- <i>O</i> - α -D-Glucopyranosyl-D-fructose
F_4	Sucrose	2- <i>O</i> - α -D-Glucopyranosyl-D-fructoside
S_2	Maltose	4- <i>O</i> - α -D-Glucopyranosyl-D-glucose
S_3	Nigerose	3- <i>O</i> - α -D-Glucopyranosyl-D-glucose

Isolation of Sucrose

The sucrose content of well-ripened honey (as determined by mild acid hydrolysis) is usually less than 1% and may be around 0.5% when analyzed after invertase hydrolysis. Actually, the content may be even lower, since at least one trisaccharide (maltosylfructoside) reported in honey (4) is split to reducing sugars by yeast invertase. This has been discussed by Täufel and Reiss (16) who could not demonstrate sucrose in 9 of 12 honey samples examined. Sucrose cannot be shown by paper chromatography in the presence of turanose, except by two-dimensional migration with invertase treatment between the migrations. Such treatment showed that only traces of sucrose were in the disaccharide sample examined. A 20.5-mg. sample of fraction F_4 was found by yeast invertase hydrolysis to contain 0.57 mg. sucrose (2.8%). This amount was not detectable in the infrared spectrum of the fraction.

The procedure described by Adcock (17) was applied to 186 mg. of mixed honey disaccharides. Reducing sugars were converted by boiling with sodium carbonate to acidic products and removed by ion-exchange treatment. The residue of nonreducing sugars (25 mg.) gave five bands on preparative paper chromatography. A nonreducing ketose band migrating with sucrose was isolated and the infrared spectrum of its octaacetate was determined. The acetate was recovered from the KBr disk and converted to the free sugar. The acetate and free sugar spectra corresponded to those of sucrose octaacetate and sucrose. It was calculated from intensities of the infrared bands that the disks contained 2.1 mg. sucrose octaacetate and 0.82 mg. sucrose, respectively. Table IV lists the disaccharides isolated from honey in this investigation.

ACKNOWLEDGMENTS

The infrared spectra were determined by Carl T. Leander. The participation of Jeanne M. Petty in the early portion of the work is acknowledged with thanks.

SUMMARY

Six disaccharides have been isolated from honey and identified by their infrared spectra and those of their β -octaacetates as isomaltose, maltulose, turanose, nigerose, maltose, and sucrose. The first four have not previously been identified in honey.

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